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Unconventional Pathogen Countermeasures
Quarterly Report

Reporting Period: 03/31/2002

Mortality and Morbidity Avoidance/Reduction of respiratory sickness immediately following exposure to bioweaponized microbial pathogens

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Abstract

Objective: This proposal seek to develop: (1) an effective broad-range formulation cocktail for pulmonary tract delivery as a countermeasure to inhaled microbial pathogens. The formulation will be polyvalent in both targeting and mechanism of action to enhance potency against multiple organisms and to prevent bioengineering to resist the formulation. (2) Bivalent components of the cocktail will be constructed by coupling targeting molecules to a variety of enzymes active against bacterial cell walls. Targeting compounds include, but are not limited to, unique inhibitors of a ubiquitous bacterial transport system important for virulence recently developed by the investigators. (3) The formulated cocktail will be compatible with self-administration upon exposure location prior to or after actual or suspected inhalation of pathogens. Chemical formulation of the cocktail will be compatible with current delivery vehicles such that the countermeasure could be deployed soon after demonstration of efficacy and approval of the formulation by the necessary regulatory agencies. Threat organisms treatable by the proposed novel unconventional countermeasure include, but are not limited to: spores and vegetative cells of Bacillus anthracis, Clostridium spp., fungal lung pathogens in general and cells of Yersinia pestis, Francisella tularensis, Brucella suis, Salmonella typhi and related bacterial species.

Background

1. TECHNICAL RATIONALE

Nations and terrorist organizations with offensive biological warfare (BW) programs have a marked preference for aerosol dispersal of bioweapons because of its relative ease and effectiveness. This method of delivery demands several properties from a potential bioweapon agent. It must be sufficiently robust to withstand the dispersal detonation or spray. It must be easy and quick to cultivate with a minimum of infrastructure and expense. It must be stable enough for medium- to long-term storage prior to deployment to allow for stockpiling in useful quantities. It must act sufficiently fast and be lethal enough by the inhalation route that a small quantity can be dispersed over a wide area of coverage, so as to inflict the greatest number of military or civilian casualties within a militarily or politically useful time frame (i.e., days). Unfortunately there are a variety of "classical" BW agents that fulfill these requirements, including the etiologic agents of anthrax (Bacillus anthracis), plague (Yersinia pestis), typhoid fever (Salmonella typhi), brucellosis (Brucella suis and other species) and tularemia (Francisella tularensis).

These BW agents are bacterial and share several properties in common:
Successful infection by the inhalation route requires either phagocytosis by or invasion of alveolar macrophages and dispersal to the regional lymphatic system. Physical robustness and survival of the bacterium during infection is dependent upon the peptidoglycan cell wall. All of these bacterial species (and others) constitutively express a conserved magnesium transporter CorA, which is bound by the competitive inhibitor compounds cobalt(III)hexaammine and cobalt(III)chloropentaammine.

What are the implications of these shared properties?
Infection route: During infection by the inhalation route the alveolar macrophage is the site of germination by anthrax spores and the site of proliferation by bacteria of the genera Salmonella, Yersinia, Brucella, and Francisella. Proliferation within and escape from the macrophage allows systemic spread, resulting in the septicemia and release of toxins that cause the disease symptoms and concomitant morbidity and mortality. Thus any successful broad-range countermeasure to an inhaled BW agent must either attack the microbe in the pulmonary tract prior to uptake in the macrophage, or must follow the microbe into the macrophage compartment to complete destruction of the organism before it proliferates and

escapes.

Chemical nature of the bacterial cell wall: The peptidoglycan cell wall layer of bacteria is composed of a chemically distinct glycan polymer repeat of N-acetyl glucosamine linked b-1,4 to N-acetyl muramic acid, stitched together into an elastic superstructure by flexible pentapeptide bridges. These "stem" peptides form amide crosslinks between some of the muramic acid moieties during incorporation of new glycan strands into the cell wall matrix. However the peptidoglycan layer differs somewhat between different species of bacteria. Firstly there are two major differences in cell wall organization, that of Gram-positive bacteria (e.g. Bacillus spp.) and that of Gram-negative bacteria (e.g. Yersinia spp.). The cell wall of Gram-positive bacteria is externally exposed outside of the single bacterial cell membrane and tends to be heavily crosslinked and thickened into multiple sheets to provide a protective layer and mechanical rigidity. In contrast the Gram-negative bacteria organize their cell surface into a two-membrane system with a much thinner (but less surface-accessible) peptidoglycan layer sandwiched between the outer and cytoplasmic membranes. Secondly, the exact composition of the amino acid residues within the stem pentapeptides varies between species, especially among the Gram-positives. Thirdly, some Bacillus species express an N-acetylase which removes, after synthesis, a substantial portion of the N-acetyl decorations from the N-acetylglucosamine and N-acetylmuramic acid substituents of the glycan chain. In B. cereus cell wall glycan, this de-N-acetylated fraction can reach 70% of glucosamine residues, while the cell wall of B. anthracis has been reported to comprise 88% unsubstituted glucosamine and 34% unsubstituted muramic acid. De-N-acetylation of the glycan chain has not been reported for the cell walls of Gram-negative bacteria.

Gram-positive bacteria (but not Gram-negatives) also incorporate teichoic and/or teichuronic acid molecules into their cell walls adjacent to the outer surface of the cytoplasmic membrane. These molecules are thought to help regulate the activity of cell wall hydrolases called "autolysins" which typically cleave the pentapeptide crosslinks of the peptidoglycan cell wall during cell division and thus allow separation into two daughter cells. Autolysins are present not only in the genomes of Gram-positive bacteria, but also in those of Gram-negative species. For example there are several genes in Escherichia coli (lytA, lytB and amiA) which encode enzymes with autolysin activity. Close homologs are present among the other related Gram-negative pathogens such as the Salmonellae and the Yersiniae. In addition to allowing cell division, autolysins have further developmental roles for endospore forming Gram-positive bacteria. For example bacilli of the genus Bacillus utilize two sets of highly specific and tightly regulated endogenous autolysin-like enzymes to weaken the cell wall at critical steps in the developmental program. The chemical nature of the peptidoglycan differs slightly in the endospore from that of the mother cell, consequently one set of autolysins serves to release the mature endospore from the mother cell while the other set functions later during germination to release the "germling" from the endospore peptidoglycan.

The bacterial cell wall is chemically distinct from any polymers found in mammals because of the presence of (N-acetyl)-muramic acid, D-amino acids and often diaminopimelic acid. Thus the bacterial cell wall constitutes an extremely suitable target for antibacterial compounds - not only because of its surface location and essential nature, but also because of the limited side effects to mammals exhibited by compounds targeted against this unique chemistry. However, to date the only antimicrobial compounds employed medicinally to affect some aspect of cell wall physiology are penicillin derivatives and cycloserine, both of which inhibit new cell wall synthesis. Penicillins bind to and inhibit the proteins which catalyze the formation of the "stem" pentapeptides, while cycloserines inhibit the enzyme which racemizes L-alanine into the D-alanine form common to all stem pentapeptides. Crucially, both of these antimicrobials require active growth by the bacterium because they rely upon the continuing activity of endogenous autolysins to weaken the fabric of the existing cell wall while their inhibitory activity prevents the formation of extensive crosslinking between newly synthesized glycan strands.

Lysozyme, a cell wall hydrolytic enzyme. Lysozyme is an antimicrobial component of tears and saliva. It is also present as a non-specific host defense in the mucus layer of the mucous membranes, especially in the upper respiratory tract and in phagocytic cells and lysosomes. Lysozyme is also naturally present in milk to retard spoilage and in the albumin fraction of avian eggs. Most lysozyme used in the laboratory to facilitate digestion of the bacterial cell wall originates from hen eggs (a relatively cheap and abundant source). These lysozymes are typically "classical", i.e., muraminidase/chitinase, which cleave between the N-acetyl glucosamine and the N-acetyl muramic acid substituents of the bacterial peptidoglycan and between the N-acetyl glucosamine repeats of fungal chitin. In contrast, the autolysin-type enzymes typically have an amidase or glucosaminidase activity.

Why have lysozymes not been harnessed medicinally as antimicrobials? Firstly, these enzymes are only suitable for administration to mucosal or skin surfaces. Lysozymes administered intravenously would be inactivated rapidly by the immune system, while lysozymes administered orally would not survive passage through the stomach. Secondly, these enzymes are typically of low specific activity and low stability at ambient temperatures. Thirdly, Bacillus spp. of the B. cereus group (which includes B. anthracis) contain highly unsubstituted glycan chains as previously mentioned. This lack of N-acetyl substitution renders B. anthracis vegetative cells essentially resistant to hen egg lysozyme. While spore cortex peptidoglycan is potentially susceptible to lysozyme digestion, spore coat proteins normally limit access of this enzyme to its substrate. Gram-negative bacteria are not readily lysed by mammalian or avian lysozymes because of the protection afforded by the outer membrane external to the peptidoglycan layer and the low specific activity of that fraction of lysozyme which reaches its substrate. Efficient lysis of Gram-negative bacteria requires the disruption of outer membrane integrity by detergents or polycationic amines such as Polymyxin B or polylysine. Not withstanding these limitations, there has been one patent application approved in recent years for a mixture of lysozyme and surfactant to be applied to the lungs, presumably for the treatment of cystic fibrosis patients. Polycationic amine antibiotics such as Colistin (Polymyxin E) have also found renewed use delivered by aerosol or nebulizer for treatment of Pseudomonas infections of the respiratory tract.

There is however another source of lysozyme that has been totally overlooked in terms of biotechnology until recently. Bacteriophages are obligate viral parasites of host bacteria, upon which they grow, either lytically or by lysogeny (establishment of a dormant state). In either case under suitable conditions the infecting phage begins replication of new viral genomes and directs synthesis of new phage protein components. Once these new components have been assembled into progeny phages they must be released from inside the cytoplasm of the infected bacterium. With the notable exception of the filamentous bacteriophages (e.g. M13, fd), all other known bacteriophages cause the mechanical disruption and lysis of their host bacterium. Synthesis of a bacteriophage lysozyme specific for the chemical modifications (or lack thereof) of the peptidoglycan present in the cell wall of their host bacterium allows the mature virions to escape from inside the cytoplasm of their host. The bacteriophage lysozyme (endolysin) is able to reach the peptidoglycan layer on the other side of the cytoplasmic membrane due to the co-expression of a bacteriophage "holin" protein which forms a non-specific pore in the cytoplasmic membrane. "Holins" themselves have an undesirable non-specific cytotoxicity because of their innate pore-forming ability in all biological membranes. However in the context of deploying phage endolysins as a countermeasure to BW agents, we will be using endolysins as "exolysins" from the outside rather than from the inside of the bacterium, so it will not be necessary to harness the "holin" partners. Polycationic agents that disrupt the Gram-negative outer membrane will however be necessary within the countermeasure formulation.

Endolysins are not only specifically adapted to the peptidoglycan of their cognate host bacterium, but they also display a higher innate specific activity than avian or mammalian

lysozymes. For example, the endolysin of bacteriophage l has a 200-fold greater specific activity than hen egg lysozyme for the peptidoglycan of the phage's host Escherichia coli. While endolysins have the highest activity against cell walls of their host bacteria and closely related species, they also degrade peptidoglycans of many other species, albeit at a slower rate (Loessner paper, see Attachment). Since bacteriophages have been isolated for almost every bacterium, there is a great and as yet untapped diversity of phage endolysins that could be harvested for the purpose of an anti-BW countermeasure. The mixture of a diverse assortment of host-specific endolysins, each with a high specific activity against its cognate substrate, would be expected to achieve broad-spectrum protection against a variety of different BW pathogens, even if these were delivered simultaneously.

 Phage therapy: Why not use intact viable bacteriophages per se as a countermeasure to a BW organism? Indeed, "phage therapy" of bacterial infections has been revisited in recent years (especially in Republics of the former Soviet Union) as conventional antibiotics have become less useful to medicine. Firstly, many temperate phages of pathogenic bacteria can lysogenize and then express bacteriophage-encoded genes such as toxins and agglutinins that promote the virulence of the host bacterium (e.g. cholera toxin, shiga toxin, diptheria toxin), making them unpredictable and unsafe pharmaceutical tools. Secondly, the lytic effect of a bacteriophage is a combination of the holin and endolysin activities whose synthesis is reserved for very late during lytic replication. Interference in the bacteriophage life cycle, at any of several key steps prior to the production of endolysin, would prevent the lysis of the host bacterium. Thus a BW organism could be simply engineered to resist infection and destruction by particular bacteriophages.

 Targeting the highly conserved magnesium transporter CorA. Targeting additional components of the microbial cell envelope besides those to be degraded will serve to increase the efficiency of the autolysin/endolysin hydrolases. Very large increases in efficiency per unit of antimicrobial arise from a pharmacological property that increases the effective concentration of the antimicrobial at the site where it is needed if there are additional binding sites available nearby. This phenomenon occurs because release of the bivalent compound from one of its two binding moieties is followed by a high probability of reattachment because the antimicrobial remains tethered to its other binding moiety. In contrast, once a monovalent compound is released from its binding moiety, it will rapidly diffuse away and only rebind to a target after a significant period of time. This is kinetically far less favorable and thus much less probable. As digestion of the cell wall proceeds, more of the peptidoglycan matrix is opened up for attack by additional molecules of lysozyme conjugate. Thus successful lysis of the bacterial vegetative cell results from weakening of a patch of cell wall in a process analogous to rusting of metals, rather than total uniform degradation.

 We have chosen the Bacterial Mg2+ transporter CorA as a tethering target because of the extensive previous research on this transporter conducted in the Principal Investigator's laboratory. The corA gene, highly conserved among all groups of bacteria currently known (including all bacterial threat organisms), is the primary transporter of magnesium ions into bacterial cells. Fully hydrated magnesium ions associate with a domain of the CorA protein that is completely exposed on the outer face of the cytoplasmic membrane, and are then transported into the cytoplasm with accompanying dehydration of the magnesium ion. CorA is so named because mutation of the gene yields Co2+ resistance since high levels of exogenous cobalt ions are toxic to a bacterium if transported into the cell. Ni2+ is also a substrate for transport, and radiolabeled 63Ni2+ can be used to assay magnesium transport because radiolabeled magnesium is not commercially available. Research in the P.I.'s lab has shown that cobalt(III)hexaammine and cobalt(III)chloropentaammine are competitive and very selective inhibitors of the transport of radiolabeled nickel ions by CorA). Ammines are covalently bonded ligands distinct from amines and ammonium salts. The very stable cobalt(III)hexaammine and pentaammine conjugates resemble a hydrated magnesium ion but

are not transported because they cannot be dehydrated during the transport reaction. The ammines of cobalt(III)hexaammine and especially the chloride group of cobalt(III)chloropentaammine are available for reaction with a monodentate coupling ligand. For example, 1-amino, 5-azopentane contains an amino group that can substitute for an ammine ligand on the cation ammine complex, and the azo group at the other end of the pentane linker may be used for covalent linkage to various different lysozyme-type hydrolases. Multiple other secondary coupling ligands such as the sulfhydryl group could also be used to tether the degradative enzymes In this manner a chemical cocktail of bivalently targeted antimicrobials could be synthesized for use against the vegetative cells of all bacteria.

The affinity of a cobalt (III) pentaammine conjugate for the constitutively expressed CorA protein could be exploited to promote attack of the bacterial peptidoglycan layer by lysozyme. Binding of a cobalt (III) pentaammine-lysozyme antimicrobial to the CorA protein, exposed on the outer face of the cytoplasmic membrane under all physiological conditions, would concentrate the hydrolytic enzyme against the nearby peptidoglycan substrate. This substrate would now be subject to multiple attacks by a single antimicrobial molecule in repeated cycles of 'bind-hydrolyze-release' because it would still be bound to the CorA moiety. Increasing the effective concentration of the enzyme formulation through targeting-tether technology would allow lower concentrations of countermeasure to be administered to the pulmonary tract, reducing the likely incidence of adverse effects such as edema or cytotoxicity. Concentration of the antimicrobial into proximity with the bacterial cell wall would also allow the reagent to accompany bacteria into the vacuolar compartment of phagocytosing macrophages, where the bacteria could continue to be weakened further by the co-localized tethered lysozyme and inhibition of the CorA virulence factor.

Alternative targeting molecules for tethering to endolysins. It is unlikely that bacterial spores will be bound by a cobalt (III) pentaammine-lysozyme conjugate since they lack an active metabolism and are encased in a refractory spore coat. However there are other surface exposed molecules which could be targeted, such as carbohydrates recognizable by lectins, and proteins recognizable by antibodies. Consequently, we will investigate other targeting conjugates in addition to cobalt (III) pentaammine derivatives, such as lectins or single chain antibodies, which have the capability of recognizing receptors on the spore surface. Lysozymes of various kinds may be tethered to these targeting molecules so that attachment to the spore surface in the pulmonary tract allows the antimicrobial to follow the spore into the alveolar macrophages, where the spore will begin germination. At the point of germination the nascent vegetative bacillus, with a newly synthesized and thus immature cell wall, will be highly sensitive to the tethered lysozyme or endolysin antimicrobial waiting for it in the same compartment. Any cobalt (III) pentaammine-conjugated lysozyme that had also penetrated to the macrophage could at this point also begin to assault the vegetative bacillus, since CorA protein would again be surface accessible.

Summary. There is no single targeting molecule, tethered to any single variant of lysozyme, which could meet the need for prophylaxis or treatment against a wide range of threat organisms. We therefore aim to assemble a broad variety of muraminidase, glucosaminidase and amidase-type enzymes from a diversity of bacteria and bacteriophages closely related to threat organisms. Each of these enzymes will individually be covalently coupled by tethering to each variation of targeting molecule. In this manner a broad-range activity of the chemical formulation will be built up from the highly specific activities of each substituent component. The countermeasure would be self-administered into the pulmonary tract (by aerosolization) to attack both growing and non-growing bacteria, both vegetative forms and spores (upon germination) from any of the most likely threat organisms. Destruction of a large fraction of threat organisms at or near the pulmonary mucosae will reduce the infectious potential of the bioweapon by maintaining the essential sterility of the lower pulmonary system and alveolar macrophages, thus preventing or reducing the mortality and morbidity of serious systemic

disease acquired by inhalation. This novel countermeasure strategy will allow the preservation of both the lives and the combat effectiveness of combat and support personnel in and around the combat zone in the event of biological weapon deployment by an aggressor. This strategy would also have potential for protection of military and civilian populations against bioterrorist type attacks. Finally, there would be secondary commercialization possibilities for a variety of infectious diseases that might be of benefit to the general population.

Technical Approach

2. TECHNICAL APPROACH
Phase I: Identification and testing of candidate lysozymes.
 a. Clone candidate lysozyme genes with known nucleotide sequence
 Where the sequence of a suitable autolysin or endolysin gene is known and in the public domain we will design and employ specific primers for amplification using the Polymerase Chain Reaction (PCR). The amplified products will then be cloned into custom-built plasmid expression vectors that use temperature sensitive repressor proteins and the strong Pant promoter from the phage P22 ImmI region for thermoinducible expression in an E. coli host. Thermoregulation of lysozyme production is desirable so that a high cell density can be reached before synthesis of the lysozyme is rapidly initiated by an abrupt temperature upshift. Since a small portion of the cells in any actively growing culture are lysing at any given point in time, if lysozyme were to be expressed constantly from a plasmid construct this would result in premature killing of the culture before high levels of product were produced. While commercially available vectors are available for protein expression, these vectors require induction of protein expression by relatively expensive inducer compounds such as isopropyl-b-D-thiogalactoside (IPTG) and are covered by research-only licensing agreements. Since we eventually aim to produce large quantities of product and engage in other pseudo-commercial activities, these vectors are best avoided in favor of our own technology that is more suitable for fermentation scale-up.
 Purification of proteins will be accomplished by affinity tag purification. We will initially determine whether amino- or carboxy-terminus tagging is more appropriate for lysozymes and investigate whether standard tag sequences such as 6X-His are tolerated in our system, along with choice of cleaving sequence for post-purification removal of the affinity tag. Building our own proprietary vector will allow us to maintain flexibility in resolving these issues.
 Many candidate lysozymes could be directly cloned for expression by this method, including but not limited to: the endolysin of Salmonella bacteriophage P22, autolysins lytA, lytB and amiA of E. coli, the cell wall hydrolase (cwl) autolysins of Bacillus cereus and other Bacillus spp., and the phage endolysin genes from B. cereus phages TP21 and 12826.
 b. Clone candidate lysozyme genes with unknown nucleotide sequence
Phage lysozymes and endolysins: The small size of bacteriophage genomes (typically 40-170 kilobases) makes it possible to efficiently clone bacteriophage endolysin genes with unknown sequence. Partial endonuclease digestion of DNA purified from bacteriophage particles will be used to construct random clone libraries in the thermoinducible expression vectors described in Section 2a above. Individual clones from the random library will contain approximately 4 kilobases of DNA insert and can be screened for endolysin activity against an appropriate bacterial strain. Typically fewer than 100 colonies would need to be screened per library to find a clone with endolysin activity. This type of screening is readily adaptible to a high-throughput microplate assay. Sequencing of appropriate clones would then facilitate the cloning of endolysins for direct expression by the PCR method already described in Section 2a.
 Several endolysins of particular interest could be cloned and identified using this protocol. Bacillus phage CP51 is capable of forming large plaques on strains of B. cereus, B.

thuringiensis and B. anthracis and is therefore likely to express an endolysin with very high specific activity against unsubstituted peptidoglycans (i.e., de-N-acetylated forms). The Salmonella phage Ffm has an extremely active lytic ability against mutants of Gram-negative enteric bacteria that express 'rough' mutant forms of lipopolysaccharide and so is likely to express an endolysin with extremely high specific activity against a wide range of N-acetylated peptidoglycans.

c. Harvesting of crude protein extracts and affinity purification of lysins.

Prior to culturing of recombinant lysozymes, each expression construct will be sequenced through the clone region to verify sequence of the candidate gene. This is necessary because mutations are occasionally, though rarely, introduced during amplification by PCR. Small volume cultures (50 ml) will then be prepared and expression of recombinant proteins induced by temperature shift of cultures once mid-logarithmic phase growth is reached. Proteins will be harvested from late logarithmic phase cultures by centrifugation and resuspended bacterial pellets lysed in a French pressure cell. This method of mechanical disruption avoids the use of lysozyme to burst cells since this would contaminate our preparations prior to testing for efficacy. The crude extracts resulting from passage through the French pressure cell will be poured over an affinity chromatography column (e.g. Nickel-NTA for 6X-His tags). The recombinant proteins will stick to the matrix of the column while other proteins continue to flow over the column. After washing to remove proteins from the column which have stuck nonspecifically, bound recombinant proteins will be eluted from the column, producing 95-98% pure protein in a single step. The preparation will then be treated with a specific protease to cleave away the tag sequence (e.g. Factor Xa protease) and passed over the affinity column once again. This time the tag sequence will stick but the released recombinant protein will flow freely through the column. Alternatively, for short tag sequences, a simple sizing column would separate the two peptides. In our experience, this 2-step procedure typically produces 10-20 mg of 98-99% pure protein from 500 ml cultures. Such purity is sufficient for all preliminary testing required. Eventual formulation of a cocktail may require additional purification, but a second pass through the Nickel-NTA affinity column before cleavage of the tag typically produces >99% pure protein.

d. Testing recombinant enzymes for lytic spectrum and specific activity

Purified recombinant lysozyme preparations will be examined for purity by SDS-PAGE electrophoresis. Suitable dilutions of the enzyme preparations would be made to test each for the range of organisms whose cell walls it can lyse (i.e., lytic spectrum) and to determine the specific activity per unit enzyme against its preferred substrate bacteria. Lysozyme activity is most simply assayed by observing the loss in absorbance at 600 nm over time from an initial value of 1.0 OD units. This loss of absorbance represents the lysis of viable bacterial cells due to loss of integrity of their cell walls.

Tester strains of bacteria used in this assay will be surrogates of actual threat BW organisms. For example Salmonella typhimurium and Bacillus cereus could be safely used to assay potential activity of recombinant lysozymes against such BW pathogens as Salmonella typhi, Yersinia pestis and Bacillus anthracis. Laboratory strains of Escherichia coli, Bacillus subtilis and Bacillus cereus incubated with commercially available hen egg lysozyme will be used as control benchmarks to measure relative performance of the recombinant lysozymes against the N-acetylated cell walls of Gram-negative and Gram-positive bacteria respectively. Hen egg lysozyme will show little or no lytic activity against the B. cereus strain. It is to be anticipated that lysozymes with good lytic activity against de-N-acetylated cell walls (such as those of the Bacillus cereus cluster) will not perform well against the E.coli and B. subtilis standards, but will perform excellently against B. cereus.

Polycationic amines such as the Polymyxin B nonapeptide will be investigated at this point to confirm whether their inclusion in the formulation would enhance or reduce activity of any candidate lysozyme against any particular BW threat pathogens. Any performance gains would

be expected against cell walls of Gram-negative bacteria due to disruption of the barrier properties of the Gram-negative outer membrane by the polycations.

e. Testing of a poly-lysozyme formulation.

It will be necessary to identify which lysozymes, from among the collection of recombinant preparations, are most suitable for mixing into a combined formulation to achieve the best combined lytic spectrum with the least number of component enzymes. The assays described in Section 2d above can be modified by the mixing of Gram-negative with Gram-positive bacteria (both N-acetylated and de-N-acetylated forms) in equal measure (i.e., one part each Salmonella, Bacillus subtilis and Bacillus cereus). These bacterial mixtures will then be attacked with mixtures of different recombinant lysozymes combined rationally so that there will be enzymes capable of attacking each kind of cell wall within the formulation.

Phase II: Targeting molecules and tethering technologies

f. Confirm inhibition of CorA transport by cobalt(III)chloropentaammine.

Our original data demonstrating competitive and specific inhibition of CorA transporters by hexaammine and chloropentaammine derivatives of cobalt (III) was generated with Gram-negative bacteria and Archaea. While we fully expect that these compounds will also inhibit CorA transporters of Gram-positive bacteria, we will need to demonstrate competitive and specific inhibition in representative organisms such as Bacillus spp. Transport of magnesium ions is determined by measuring the uptake of surrogate radiolabeled nickel ions into the insoluble cell fraction over time, in the presence of varying concentrations of cobalt(III)hexaammine or chloropentaammine. The dose response curves generated from these experiments should show decreasing uptake of radioactive nickel as the concentration of hexaammine or pentaammine increases. Demonstrating inhibition of the CorA transporters of Gram-positive bacteria will allow us to employ cobalt (III) pentaammine as a targeting molecule against vegetative cells of all the threat bacteria, including anthrax.

g. Synthesis of tethered derivatives of the best candidate enzymes.

Once candidate lysozymes have been identified in section 2d (above) and pass screening for lack of cytotoxicity in section 2k (below), they will be derivatized by tethering to the targeting molecule cobalt(III)chloropentaammine. Coupling will be achieved by reaction of the chloride group of the chloropentaammine with a monodentate ligand, such as 1-amino 5-azo pentane, which leaves a free azide group. This reactive center can now be used to couple the targeting molecule covalently to each variant of lysozyme as it passes testing in stages 2d and 2k. Other coupling chemistries will also be investigated to allow conjugation of other targeting molecules besides cobalt(III)chloropentaammine.

h. Investigation of alternative targeting molecules.

Since CorA protein is not likely to be available for targeting in bacterial spores, other targeting molecules must be investigated that do recognize surface components of the spore. Types of molecules that may prove useful for these purposes are lectins and single chain antibodies. Receptor molecules on the spore surface for these compounds include carbohydrate moieties (lectins) and proteins or peptides (single chain antibodies). It is likely that this aspect of targeting will need to be highly specific to ensure effective targeting of the spore surface while avoiding binding to the surfaces of human cells. Lectins have already be used by another group to differentiate between spores of the closely related Bacillus anthracis and B. cereus, giving us confidence that some aspect of this technology can be employed successfully. There is a strong likelihood that we will need to work with actual threat organisms at this point in the research program because cell surface components are highly variable between species, meaning that countermeasures targeted to a surrogate organism might not be effective against an actual BW pathogen. All handling and experiments involving actual BW pathogens will be conducted in a BL3 facility, and we will endeavor to obtain toxin minus strains for additional safety of personnel. Once effective alternative targeting molecules have been developed, these will be tethered to candidate lysozymes, which is why we have to develop several alternative

methods of covalently tethering compounds, as described in section 2g above.

i. Comparison of specific activity and lytic spectrum of tethered enzymes.

Enzymes tethered to targeting molecules (steps 2g and 2h above) will be evaluated to confirm that they retain lytic activity against the correct organisms using the assays already described in 2d and 2e above. At this time we also anticipate noting increases in the apparent specific activity of each tethered lysozyme component due to the performance gains expected from the enhanced effective concentration properties of tethered compounds. Components that pass this testing phase will be re-examined for cytotoxicity (step 2l below).

j. Test tethered compounds for bactericidal action from within macrophages.

Critical to the effectiveness of our countermeasure formulation will be the ability of targeted compounds to bind to vegetative bacteria and spores prior to engulfment by macrophages. This allows the lysozyme component to attack the bacteria during proliferation or germination (for spores) within the phagocytic cells. Variable concentrations of individual tethered lysozymes will be mixed with appropriate surrogate threat organisms, which have been opsonized where necessary, immediately prior to mixing with cultured macrophage cell lines and primary cultures of alveolar macrophages.

These phagocytosis assays will be allowed to proceed over time and, after washing away unphagocytosed pathogens, samples will be removed at suitable time points, e.g. every hour. Each sample will be processed with 0.1% Triton X-100 to gently lyse the macrophages. Supernatant fractions will be plated for viable counts of bacteria on media suitable for supporting the growth of the pathogen, in most cases, simple Luria Bertani (LB) agar. If the lysozymes are effective, we expect to see a reduction in bacterial viability with increasing time, while untreated controls will instead show a proliferation in the numbers of viable bacteria. Once minimal inhibitory concentrations (MIC) have been determined for each individual component, the performance of the whole countermeasure formulation will be determined using assays similar to those described above, but also testing activity against mixtures of pathogens as well as pure cultures.

Phase III: Toxicity testing of formulation components.

k. Testing individual components for lack of cytotoxicity.

Individual lysozyme type antimicrobials will be tested for lack of cytotoxicity against cultured macrophages of both murine and human origin (in the absence of bacteria). The first screen will be of untethered lysozymes so as to avoid unnecessary tethering syntheses of cytotoxic proteins to targeting molecules. Of course the targeting molecules which will be tethered to the lysozymes must also be screened for lack of cytotoxicity to these same cells. Preliminary data that we have generated previously indicates that cobalt(III)hexaammine and its derivatives are not cytotoxic to cultured mouse macrophages.

l. Testing cytotoxicity of tethered bivalent compounds and polyvalent mixtures.

Once individual lysozymes and targeting compounds have passed the cytotoxicity screen described in 2k above, the tethered bivalent compounds will be synthesized and tested for activity (see 2g through 2i above). Each variant of tethered bivalent compound must then be evaluated for lack of cytotoxicity using the same screen described in 2k. Bivalent compounds that pass this screen are then candidates for inclusion in a polyvalent lysozyme mixture. This final polyvalent countermeasure formulation, whose composition is determined by efficacy testing, must also be tested for cytotoxicity using cultured cell lines as described in 2k above.

m. Testing of countermeasure formulation for toxicity and edema in small animals.

Having arrived at a final countermeasure formulation which demonstrates efficacy against a broad range of surrogate organisms, and which lacks cytotoxicity in vitro, it will be necessary to test the formulation for adverse events in a whole animal model. Section M, Vertebrate Animals (below) describes our full animal protocol. The countermeasure will be delivered to small batches of uninfected healthy 7 week-old BALB/c female mice by intravenous, intraperitoneal, oral gavage and inhalation methods. The mice will be examined for indications such as toxicity,

gastrointestinal distress and pulmonary edema so that the effects of administering the countermeasure to healthy individuals can be estimated. Some of these complications might be lethal to the mice, while others might necessitate sacrifice of the mice. Mice that survive for two weeks without symptoms will be sacrificed and autopsied for occult indications. Post mortem services are available from N. Kleinman, D.V.M. the Assistant Director of the institutional Animal Resource Facility. Obviously, the most critical data concerns adverse events due to the inhalation of our countermeasure, the other methods of administration are included to gather preliminary information about the consequences of drug misadministration.

In the event that toxicity or edema is observed, especially if by the inhalation route, it will be necessary to split the formulation down into components. Each of these will then be tested in the whole mouse model to determine which causes the adverse effect(s). If the countermeasure formulation were to induce pulmonary edema, this symptom might be prevented by the co-administration within the formulation of a corticosteroid, such as dexamethasone, to prevent inflammation. Otherwise, re-engineering of the formulation will be necessary with selection of replacement components from among the redundant variations discovered in 2d, Phase I above. Another round of toxicity testing will be conducted with the replacement compound alone, which if successful will be followed by toxicity testing of the newly reformulated countermeasure.

Phase IV. Efficacy and prophylaxis of countermeasure using animal models of disease.

n. Establishment of effective dose, pre-exposure dosing.

Once a countermeasure formulation which is both effective and non-toxic has been identified in Phases II and III above, it will be necessary to demonstrate that it has protective ability against inhaled pathogens in a mouse model of disease. BALB/c mice are highly sensitive to Salmonella typhimurium administered by the inhalation route. The disease progression is very rapid and animals are clearly sickened and often prostrate or dead within 24 hours after inoculation by the inhalation route. As an initial experiment the countermeasure formulation will be administered to the lungs of mice, immediately prior to exposure to lethal and sub-lethal doses of inhaled pathogens. This will allow estimation of the dose necessary to protect an individual whose exposure to BW agents is expected or imminent. Different methods of delivery and dry powder versus liquid formulations may be evaluated at this stage for efficacy.

Salmonella typhimurium in mice is a good model for Gram-negative pathogens such as S. typhi, Yersinia pestis and Francisella tularensis. A potential model for Gram-positive pathogens will be the administration to mice of Bacillus cereus vegetative cells and spores, but the quantity of organisms necessary for infectious and lethal doses may have to be determined by us experimentally. Should surrogate models not work, especially those for Bacillus anthracis, we will have to conduct this series of experiments in the BL3 facility using a guinea pig model with actual B. anthracis pathogens.

o. Establishment of effective dose, post exposure dosing.

The testing of formulation efficacy in whole animal models of disease as described in 2n (above) will be repeated with administration of the countermeasure immediately after exposure to the inhaled pathogen. This will allow estimation of the dose necessary to prevent mortality or morbidity of an exposed individual. Different methods of delivery and dry powder versus liquid formulations may be evaluated at this stage for efficacy.

p. Determination of dosing efficacy time-line.

Once the necessary doses for basic, pre- and post exposure dosing are determined, more complex investigation of the optimum dosing time frame may be established. Parameters such as the duration of effectiveness of pre-exposure dosing or the benefits and frequency of repeated post exposure dosing, may be determined.

q. Testing of formulation against actual threat agents.

After all of the delivery parameters have been determined in surrogate model systems there will need to be testing of the countermeasure against real BW threat pathogens. Experiments

will be similar to those described in sections 2n through 2p above. Experiments will be performed in the BL3 facility for personnel safety. Threat organisms to be tested include Yersinia pestis and Bacillus anthracis. At this stage in testing, which will be very late in the third year of our research plan, it may be necessary to consult and cooperate with elements of the United States Army Medical Research and Materiel Command (USAMRMC) at Fort Detrick, MD. This will allow the best and most appropriate model systems to be used for a suitable demonstration of our technology.

3. DEMONSTRATION PLAN

Demonstrations that research objectives have been achieved will be as follows:

Phase I. Demonstrations.

Demonstrate individual lysozymes with in vitro lytic activity against vegetative cells of one or more BW threat pathogens, or surrogate organisms. Demonstrate a mixture of these lysozymes that has a combined in vitro lytic activity against vegetative cells of all classes of BW threat bacteria.

Phase II. Demonstrations.

Demonstrate the construction of tethered derivatives of candidate lysozymes to various targeting molecules, including but not limited to cobalt(III)chloropentaammine. Demonstrate the enhanced in vitro lytic activity that results from tethering conjugates. Demonstrate that the lysozyme conjugates can accompany threat pathogens or their surrogates into cultured macrophages and complete destruction of the pathogen, including spore forms.

Phase III. Demonstrations.

Demonstrate, concurrent with Phase II investigations, that candidate lysozymes and their conjugated derivatives are not cytotoxic to cultured human or murine cells. Demonstrate that the countermeasure formulation or its components do not cause significant adverse events, or that adverse events can be readily controlled by additions to the formulation.

Phase IV. Demonstrations.

Demonstrate in vivo protection from (pre-exposure), or treatment of (post exposure), inhaled surrogate organisms and true BW pathogens using appropriate whole animal models of disease. Demonstrate knowledge of those parameters that affect dose concentration; timing, frequency and method of dose delivery; and the physical state of the dose.

C. PROGRESS REPORT Not applicable.

D. COMPARISON WITH OTHER ONGOING RESEARCH

Advantages of our proposed method. DARPA seeks a countermeasure to the threat of BW agents. This proposal seeks funding for research and development of a chemical formulation containing engineered enzymes that will reduce or eliminate the mortality and morbidity associated with exposure to bioweaponized microbial pathogens. The strength of our method is the broad spectrum of bioweapon agents that are potentially countered by deployment of our formulation. Thus the specific nature of a bioweapon attack does not need to be identified before the countermeasure can be deployed, from a lightweight, compact and self-administered platform.

Other BW defense research priorities include the development and deployment of accurate 'real-time' methods for detection of threat pathogens. Our countermeasure will not be rendered obsolete by advances in this field since the formulation is aimed at a broad spectrum of BW agents rather than a single BW threat pathogen, nor is our countermeasure dependent upon advances in detection methods for it to be effective. Instead, our countermeasure concept adds to the existing layers of protective measures, such as the protective clothing of MOPP protocols and current and future vaccine schedules. Significant degradation of combat effectiveness results from donning the protective clothing inherent to MOPP protocols because of the discomfort and reductions in communication and sensory perception. Ideally, our countermeasure would allow a lower level MOPP to be employed within any given BW threat scenario, thus restoring at least a portion of combat effectiveness.

An additional deterrent effect stems from the ability of most troops exposed to BW agents to survive the attack and retain combat effectiveness, even if they were not at the highest MOPP when attacked. An aggressor is likely to deliver BW munitions only if some military or political advantage results, such as a high casualty rate. If this advantage cannot be guaranteed then the threat of retaliatory strikes outweighs the benefits of a BW offensive.

The biological effectiveness of our proposed countermeasure results from direct delivery to the most likely site of infection, the surfaces of the pulmonary system, and from the ability of at least fractions of the countermeasure to accompany phagocytosed pathogens into the lymphatic system. This targeting for post-phagocytic efficacy is achieved by the tethering of targeting molecules to the enzyme active component and also yields a massive improvement in drug performance because of increases in effective concentration. Direct delivery avoids the need to build up a high systemic concentration of drug, as with regular antibiotic therapy, and the use of degradative enzymes which lyse from the outside, rather than inhibitors, allows non-growing as well as actively growing bacteria to be destroyed.

The potential to engineer BW threat pathogens to resist our countermeasure is not great. Firstly, altering the extent of acetylation of the peptidoglycan, modifying teichoic acid structure, or modifying stem pentapeptides would merely increase sensitivity of a pathogen to another lysozyme component, and would also interfere with normal biological processes such as cell division and sporulation of the bacterium. Resistance is easily engineered to Polymixin B, necessary in our formulation to breach Gram-negative outer membranes, but has the side effect of increasing sensitivity to the complement proteins in serum. Engineering organisms by deletion of the corA gene to prevent targeting by cobalt (III) pentaammine derivatives results in great attenuation of an organism's virulence (our unpublished data), due to the pleotropic effects of magnesium limitation within the bacterial cell.

Inhibition of hen egg lysozymes (muraminidase/glucosaminidase) has been demonstrated with tri-N-acetylglucosamine (tri-NAG), which binds into the active site of the enzyme but cannot be cleaved or released. This inhibitor would only inactivate a subset of the lysozyme enzymes within our formulation, and amidase type enzymes would be completely unaffected. Furthermore, engineering a BW organism to synthesize and export tri-NAG without disrupting cell wall synthesis would be technically extremely challenging.

Our general strategy also allows us to respond in the future to intelligence estimates for newly weaponized threat pathogens, including engineered variants of current non-BW pathogens. For example, engineered hyper-virulent fungal pathogens, use of aerobically growing Clostridium botulinum spores to deliver botulinum toxin to the lungs (rather than the toxin itself), or other agents such as multiple drug resistant clinical isolates of Staphylococcus or Streptococcus. Our response would be to identify and harness bacteriophage endolysins and bacterial autolysins from these organisms to supplement our countermeasure formulation, if it proved ineffective against these newly weaponized threats.

Disadvantages of our proposed method. Our countermeasure formulation can only be expected to be effective for protection against and treatment of BW inhalation threats. It is probably not suitable for gastrointestinal protection, and might not work effectively for decontamination of skin surfaces. However we believe that the correct formulation will be very effective for amelioration of the inhalation threat, and have focused the thrust of this research proposal at that goal.

Bacteria have the capacity to synthesize a loosely attached extracellular capsule, commonly composed of polysaccharide or mucopolycaccharide. In the case of Bacillus anthracis, the capsule is a known virulence factor, but is synthesized under conditions (e.g. high carbon dioxide concentration) found in specialized environments beyond the lungs. Thus we aim to have our countermeasure attached to the spore/germling long before capsule is produced. Gram-negative pathogens can also make capsule, however the oligosaccharide form of capsule is embedded in the outer membrane and thus surmountable by polycations such as

polymixin B. In contrast the 'slime' type of Gram-negative capsule e.g. E. coli colanic acid capsule, is not made at temperatures above 25C and so is unlikely to present problems during inhalation disease.

Comparison to other research. Three potential antibiotic therapies are currently in the spotlight.

Type III secretion systems. Many Gram-negative pathogens, including enteric bacteria such as Salmonella, Yersinia, and Escherichia and plant pathogens such as Erwinia and Pseudomonas, express these secretion systems for delivery of bacterial effector molecules directly into, or in proximity with, adjacent eucaryotic cells. Such effector molecules play a large role in the ability of these bacteria to cause gastrointestinal and plant blight diseases. However, inhibition of Type III secretion systems is not currently feasible because of the lack of drugs which target this activity. Furthermore, Gram-positive BW threats, such as Bacillus anthracis do not encode these systems within their genomes.

Antisense RNA. In theory, antisense RNA can be employed to prevent the translation of genes required for bacterial pathogenesis. The antisense molecule binds specifically to the messenger RNA of the cognate gene and prevents translation by the ribosomes. However, in practice, a separate incredibly specific antisense RNA countermeasure would have to be designed for every potential BW threat pathogen. In addition, methods for gaining entry of the antisense molecule into the cytoplasm of the pathogen are not efficient enough to provide either prophylaxis or treatment against a large dose of organisms.

Phage therapy. Bacteriophages may be useful for treating bacterial infections in the lungs or topically. However, attachment of phages to host bacteria requires very specific receptor interactions that vary between strains of the same species, as well as between separate species, and limit the phage host range. In addition, these bacterial viruses need to replicate inside their hosts, again this process is commonly blocked in different strains of the same species. Effective use as a BW countermeasure would therefore require detailed knowledge of the delivered BW agent, in addition to a suitably broad and immediately available pharmacopoeia of bacteriophages.

Deliverables

DELIVERABLES ASSOCIATED WITH PROPOSED RESEARCH

The research described in this proposal will generate detailed knowledge of enzymes and enzyme conjugates that are active against individual threat organisms. Data will be generated describing the efficacy, specific activity, lytic spectrum and toxicity of each of these enzymes and enzyme conjugates. In addition, the properties of a polyvalent mixture of some or all of these enzymes and enzyme conjugates will be examined for usefulness as a BW countermeasure. Thus the efficacy, specific activity, lytic spectrum and toxicity of the whole formulation will also be determined. The broad parameters by which this countermeasure can be deployed, i.e. method of administration (aerosol liquids versus breath-actuated dry powders) to the pulmonary tract and pre-exposure dosing versus post-exposure dosing, will also be investigated and prelimi-nary data generated using small animal models of disease, primarily mice. Initial formulations of the polyvalent cocktail should be available by the mid-point of year 3.

Portions of a countermeasure formulation could be parted from the whole and applied to specific diseases for civilian medical purposes. These sub-formulations may be patentable and of commercial value, without compromising the original BW countermeasure formulation. Reformulation of the polyvalent cocktail for potential civilian use and deliverables associated with civilian use will be in a time-frame past the end of the current 3 year project.

BW Targets

Threat organisms treatable by the proposed novel unconventional countermeasure include, but are not limited to: spores and vegetative cells of Bacillus anthracis, Clostridium spp., fungal lung pathogens in general and cells of Yersinia pestis, Francisella tularensis, Brucella suis, Salmonella typhi and related bacterial species.

Clinical Targets

In principle, any pulmonary pathogen could be treated by the deliverables from this project. Thus in addition to the organisms seen as bioweapon threats, such organisms as Pseudomonas aeruginosa and Burkholderia cepacia and possibly various organisms inducing pneumonias could be treated.

Tasks

3. DEMONSTRATION PLAN
 Demonstrations that research objectives have been achieved will be as follows:
Phase I. Demonstrations.
Demonstrate individual lysozymes with in vitro lytic activity against vegetative cells of one or more BW threat pathogens, or surrogate organisms. Demonstrate a mixture of these lysozymes that has a combined in vitro lytic activity against vegetative cells of all classes of BW threat bac-teria.
Phase II. Demonstrations.
Demonstrate the construction of tethered derivatives of candidate lysozymes to various targeting molecules, including but not limited to cobalt(III)chloropentaammine. Demonstrate the en-hanced in vitro lytic activity that results from tethering conjugates. Demonstrate that the ly-sozyme conjugates can accompany threat pathogens or their surrogates into cultured macro-phages and complete destruction of the pathogen, including spore forms.
Phase III. Demonstrations.
Demonstrate, concurrent with Phase II investigations, that candidate lysozymes and their conju-gated derivatives are not cytotoxic to cultured human or murine cells. Demonstrate that the countermeasure formulation or its components do not cause significant adverse events, or that adverse events can be readily controlled by additions to the formulation.
Phase IV. Demonstrations.
Demonstrate in vivo protection from (pre-exposure), or treatment of (post exposure), inhaled sur-rogate organisms and true BW pathogens using appropriate whole animal models of disease. Demonstrate knowledge of those parameters that affect dose concentration; timing, frequency and method of dose delivery; and the physical state of the dose.

Body

1. Significant or noteworthy project highlights

As in previous report periods, work has continued on two fronts: i) production and cloning of lytic enzymes and ii) synthesis of bivalent linking reagents based on Co(III)hexaammine to

target the Bacterial CorA Mg2+ receptor.

Vector construction and enzyme cloning. Because of the firing of Dr. M. Lawes and the need to have immediate production of lytic enzymes, the primary vector we were synthesizing, a temperature sensitive the mnt/arc promoter control elements of the P22 bacteriophage, was placed on hold. Cloning and production of lytic enzymes was emphasized.

We modified a standard pBAD arabinose dependent vector for production of lytic enzymes by modifying its multiple cloning sites for rapid insertion of lytic enzymes of our choice coupled to a 6X His tag with internal cleavage site. In concert with this, we had previously cloned into this vector the endolysin of the S. Typhimurium P22 phage and the TP21 autolysin from a phage of B. cereus. Although we could produce enzyme after induction with arabinose, enzyme activity appeared low initially. Optimization of culture conditions and correction of some minor cloning artifacts increased yield to acceptable levels. Cloning of addition lysins is also in progress.

The P22 phage and TP21 autolysin enzymes were tested against but Gram-negative and Gram-positive bacteria, with hen egg lysozyme as control. Hen egg lysozyme alone had no effect on growth on plates or in broth. In contrast, but phage enzymes markedly inhibited outgrowth of an initial inoculant and markedly reduced viable colony counts in saturated cultures. Dose response curves and stability were not tested further due to project termination.

Synthesis of bivalent reagents based on Co(III) hexaammine. Dr. Xuemei Zhang was hired effective Feb. 1, 2002 when her visa was finally granted. Before termination of the project in mid-March. In short order, Dr. Zhang optimized all previous synthetic strategies and produced considerably amounts of both mono- and di-substituted Co(III) ammine derivatives. She had just produced several mg of a new series of di-substituted compounds at project termination. These have not been tested against any microbes.

2. List milestones due for the reporting period and progress made toward those milestones

a. Completion of synthesis of enzyme production vector and lytic enzyme cloning.
Two lytic enzymes have been cloned into the pBAD vector, the yield optimized, enzymes purified to >90% purity and tested for activity against Gram-negative and -positive bacteria with positive results.

b. Coupling of Co(III)hexaammine derivatives to BSA and eventually to lytic enzymes.
Synthetic routes and yields were optimized for mono- and di-substituted Co(III) ammine derivatives'

3. Include charts, tales, and/or graphics that support your conclusions/discoveries

None

4. Explain potential problems/delays for each milestones and potential solutions/innovations

Not applicable because of project termination

5. Include results from collaboration with other PI's within the UPC program as well as other government agencies
NONE

6. Include filed and issued patents and publications
NONE.

7. Wherever possible include information that is relevant to the Department of Defense and biological warfare defense.

List current active support [title, agency, amount]

A. ACTIVE SUPPORT
1. Grant: NIH R01-GM39447-13 P.I. M.E. Maguire
Title: Magnesium Transport in Microorganisms
Role: Principal Investigator % Effort: 30%
Dates and Costs: 1/1/95 to 12/31/02, Direct costs = $815,000
Current Year: 1/1/01 to 12/31/01, Direct costs = $203,000
Structure-Function of CorA Mg2+ Transport Protein of S. typhimurium. Purification,
reconstitution, genomics, and role in pathogenesis. Role in pathogenesis and properties of
MgtC, a Mg2+-regulated membrane protein of S. typhimurium.

2. Grant: NIH R01-GM61748-01 P.I. M.E. Maguire
Title: Manganese Homeostasis in Salmonella
Role: Principal Investigator % Effort: 30%
Dates and Costs: 1/1/02 to 12/31/05, Direct costs total $730,000
First Year Costs: 1/1/02 to 12/31/02, Direct costs requested = $197,500
Role on Mn2+ in microbial pathogenesis and cellular resistance to reactive oxygen.

Next Steps

Next Steps: List proposed research accomplishments for next quarter:

A site visit by Drs. M. Strand and L. Parks occurred on 2/20/02. It was noted by Drs. Strand and
Parks as well as the P.I. that progress had not been good. The P.I. was requested to come to
Arlington to meet with Dr. Carney. The P.I. presented a revised experimental plan to get the
project back on track including recruiting of a new, more experienced chemist as a
Co-investigator. After discussion, Dr. Carney made the determination that the project should be
terminated effective immediately.

As an additional point, during the meeting in Arlington, Dr. Parks stated that the original review
panel and thus DARPA felt that the primary novelty of this project was the synthesis of a
Co(III)ammine derivative coupled by a long spacer element to a lytic enzyme so that the
bivalent compound so synthesized would span the periplasmic space leaving the
Co(III)ammine bound to CorA on the inner membrane and the lytic enzyme still outside the
outer membrane. This came as a complete surprise to the P.I. Previous to the 3/14/02 meeting,
no scientific feedback and especially no feedback related to the proposal's review had been
received from anyone at DARPA. The P.I.'s concept of the original proposal did not make such
a compound as Dr. Parks described a high priority but instead emphasized bivalent reagents of
several types. Because of the personnel problems encountered with Dr. Lawes and recruitment
of a chemist, it is highly doubtful that more progress would have been made had this been
previously communicated. Nonetheless, as a hopefully constructive criticism, the P.I. suggests
that DARPA consider informing P.I.'s upon initial funding of the aspect(s) of the proposal that
DARPA finds of special interest or wishes emphasized.

Transition Activities:
Commercial product development: NONE.
DoD/Government product development: NONE.

OK

For more information about this system please contact:

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[System Planning Corporation]